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PRINCIPAL INVESTIGATOR: Srinath Palakurthi, Ph.D

CONTRACTING ORGANIZATION: Texas A&M University System Health
College Station, TX 77845

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14. ABSTRACT: Aim: Impaired balance between pro-apoptotic and anti-apoptotic molecules is common in cancer cells, including breast cancer, and it plays an important role in tumor initiation and progression ¹ . Proapoptotic gene therapy aims at enhancing the capacity of tumor cells to undergo apoptosis and renders the tumors sensitive to classical anticancer drugs and radiotherapy. We developed a novel therapeutic strategy that combines both targeted gene (p53) delivery using sigma-receptor ligand-conjugated PAMAM dendrimers, followed by chemotherapy using doxorubicin. Methods: A sigma receptor-ligand, SV119, was conjugated to PAMAM dendrimers and structural analysis of the conjugate was performed using ¹ H NMR & MALDI TOF spectroscopy. PAMAM-NHS-SV119 (PAMAM-SV119) was fluorescently labeled using FITC (Fluorescein isothiocyanate) and cellular uptake study was performed in MCF-7 cells. PAMAM-GFP-p53 dendriplexes were prepared at various charge ratios (N/P) and the transfection studies were performed in MCF-7 and NCI/ADR-RES (resistant cell) cells. Following transfection, the cells were treated with doxorubicin to determine if transfection with p53 sensitizes the NCI/ADR-RES cells to doxorubicin treatment. Results & Discussion: The ¹ H NMR spectrum of the conjugate revealed the presence of peaks of aromatic ring protons of SV119 corresponding to 6.8ppm, 6.9ppm and 7.2ppm along with -CH protons of PAMAM from 2.5-3.7 ppm. Cellular uptake studies reveal that the uptake of PAMAMG3.5-SV119 was several folds higher than PAMAMG3.5 dendrimers in all the cell lines tested. Uptake of PAMAMG3.5-SV119 was significantly higher than PAMAMG3.5 dendrimers. Agarose gel electrophoresis showed that PAMAM-SV119 was able to condense pDNA at N/P ≥ 1. Transfection efficiency of pDNA-PAMAM-SV119 dendriplexes gradually increased with increase in charge ratio from N/P 10 (MCF-7: 13.65±1.25%, NCI/ADR-RES: 19.5±2.3) to N/P 40 (MCF-7: 39.98±1.5%, NCI/ADR-RES: 39.74±2.22). Co-treatment with doxorubicin and dendriplexes (N/P 10) resulted in decreased IC ₅₀ of doxorubicin from 29.66 nM to 10.94 nM in MCF-7 cells. The NCI/ADR-RES cell viability decreased from 69.44±1.34% to 56.30±0.64% at 5 μM doxorubicin and multiple dendriplex treatment (N/P 10). Conclusion: The results conclude the specificity of SV119 conjugated dendrimers to breast cancer cells. A synergistic effect was seen between p53 transfection and chemotherapy in both the MCF-7 and doxorubicin-resistant cell lines. The present study validates our hypothesis that proapoptotic gene (p53) therapy sensitizes doxorubicin-resistant cells to doxorubicin therapy.					
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Introduction:

Proapoptotic gene therapy aims at enhancing the capacity of tumor cells to undergo apoptosis and renders the tumors sensitive to classical anticancer drugs and radiotherapy. The proposed project is designed for the targeted delivery of proapoptotic genes using PAMAM dendrimers. We hypothesize that conjugation of sigma ligands to dendrimers will specifically target and deliver the proapoptotic gene (p53 plasmid) to breast cancer cells over expressing the sigma receptors.

Specific aims of the project are to: (1) characterize sigma ligand-conjugated PAMAMG4 dendrimers by NMR and MALDI-TOF spectroscopy, (2) determine the cellular uptake and tumor specificity of the surface modified dendrimers in various breast cancer cell lines and normal cells, (3) characterize the cancer cell specific expression of p53 (GFP-p53 plasmid), (4) assess the efficiency of these vectors in inducing apoptosis and sensitizing the drug resistant breast cancer cells for chemotherapy.

Body: As stated in the statement of work (SOW) the project encompasses four tasks. A progress report on the experiments performed to accomplish these tasks is given below.

Task 1: To determine the breast cancer cell specificity of sigma receptor ligands (haloperidol and ibogaine)-conjugated polyamidoamine (PAMAM) dendrimers

Poly(amidoamine) (PAMAM) dendrimers of 3.5 generation with carboxylate surface functional groups were first activated with N-hydroxysuccinimide (NHS) to obtain a stable PAMAM-NHS. This NHS activated PAMAM was then coupled with SV119. Nuclear magnetic resonance (^1H NMR) spectroscopy was performed using Bruker 300MHz spectrometer with D_2O and presence of SV119 in final product was identified.

Initially, PAMAM dendrimers of 3.5 generation was conjugated to SV119 using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling reaction. This EDC cross linker will couple COOH surface groups of PAMAM dendrimer with terminal NH_2 group of SV119 by forming an amide bond. Briefly, EDC (8.8 mg) was dissolved in 3mL of 0.1M MES [2-(*N*-morpholino) ethanesulfonic acid] buffer, pH 4.7. To this 20 mg of PAMAM dendrimer dissolved in deionized water (1 mL) was added drop wise under stirring. This solution was stirred under nitrogen for one hr at room temperature. After 1 hr, SV119 (12.8 mg) was directly added to reaction and stirred for 4 hrs at room temperature. The reaction mixture was dialyzed with 1:10 diluted MES buffer for 24 hrs and lyophilized.

The ^1H NMR spectrum of final conjugate did not show any peaks of aromatic ring protons of SV119 corresponding to 6.8, 6.9 and 7.2 ppm, only the -CH protons of PAMAM from 2.5-3.7 ppm were present (Figure 1). These aromatic peaks were found when NMR spectrum of SV119 was recorded. As the reaction pH was 4.7, the loss of aromatic ring of SV119 was attributed to low pH of the reaction.

Thus, the reaction method was then modified to a two step conjugation instead of one with formation of stable *N*-hydroxysuccinimide (NHS) ester of PAMAM as initial step. Briefly, in the first step PAMAMG3.5 (20 mg) was conjugated to NHS (5.3 mg) by EDC (8.8mg) coupling reaction in 0.1 M MES buffer. After 12 hrs of reaction at room temperature the reaction mixture was dialyzed and lyophilized. In the second step 20 mg NHS ester of dendrimer was first dissolved in phosphate buffer of pH 7.4. To this solution while under stirring SV119 was directly added and reacted for 6 hrs at room temperature. SV119 was conjugated to PAMAMG4 dendrimers using BS^3 reagent (Pierce, Rockford, IL). Briefly, PAMAMG4 (20 mg) was dissolved in deionized water followed by addition of BS^3 reagent solution at 1:10 molar ratio. The solution was stirred for 30 min followed by addition of SV119 solution (in deionized water) at 1:10 molar ratio followed by stirring for another 4 hr. all reactions were carried at room temperature. The mixture was dialyzed against 1:10 diluted phosphate buffer pH 7.4 for 24 hrs and then lyophilized.

The ^1H NMR spectrum of the conjugate revealed the presence of peaks of aromatic ring protons of SV119 corresponding to 6.8, 6.9 and 7.2 ppm along with CH protons of PAMAM from 2.5-3.7 ppm (Figure 2). These peaks were not found in NMR spectrum of the parent PAMAM dendrimers. Thus, conjugation of SV119 to PAMAM was confirmed by presence of these peaks corresponding to aromatic ring protons.

To confirm the molecular weight of SV119-conjugated dendrimers, mass spectral analysis of the dendrimers was performed on a Applied Biosystems 4700 MALDI-TOF (matrix assisted laser desorption/ionization-time of flight) using acetonitrile:H₂O; 50:50 having 0.3% TFA as a solvent and α -cyanohydro-cinnamic acid as the matrix. MALDI-TOF spectra (Figure 3) have revealed that about 8 and 12 molecules of SV119 were attached to PAMAMG4 and PAMAMG3.5 dendrimers, respectively.

SV119-conjugated PAMAM dendrimers were labeled with Fluorescein isothiocyanate (FITC). Briefly, a 5 mg/mL solution of FITC in acetone was prepared and added to the dendrimer solution in PBS, pH 7.4 at a molar ratio of 1:1 and was stirred overnight at room temperature. Fluorescently labeled dendrimers were dialyzed against deionized water and fractioned on a Sephacryl S-300 column using 30:70 (v/v) acetonitrile:Tris buffer (pH 8). The concentration of the labeled dendrimers was measured by spectrofluorometer and it was found that approximately 1.2 molecules of FITC were conjugated to one molecule of SV119-conjugated PAMAM dendrimers. The specificity of SV119-conjugated dendrimers to the various cancer cells was evaluated by comparing the extent of uptake of the native PAMAMG3.5 dendrimers (control) and SV119-conjugated dendrimers in various the cancer cell lines (human prostate cancer cell line, PC-3; human breast cancer cell line, MCF-7; ovarian cancer cell line, OVCAR-3). Either FITC-labeled native or SV119-conjugated dendrimers were added to the cells in a total of 200 μ L, in a 24 well plate and incubated for different time intervals at 37°C. The cells were washed three times with phosphate buffered saline (PBS) and analyzed for fluorescence intensity by flow cytometry. To determine the non-specific binding of the dendrimers to the cell surface, cells were incubated with the dendrimers in cold PBS at 4 °C for 2 min, washed with PBS and the fluorescent signal was measured as mentioned above. Intracellular fluorescence was calculated by subtracting the fluorescence from the cells treated with the dendrimers in cold PBS from the treatment at 37 °C. The data reveal that the uptake of PAMAMG3.5-SV119 was several fold higher than PAMAMG3.5 dendrimers in all the cell lines tested (Figure 4). Uptake of PAMAMG3.5 is very low as expected because of the net surface negative charge of these dendrimers. The higher uptake of PAMAMG3.5-SV119 may accounted for the sigma receptor mediated endocytosis by using SV119. The uptake of amine-terminated PAMAMG4-SV119 is higher than PAMAMG3.5-SV119 as their uptake is mediated both by receptor mediated endocytosis and charge-mediated endocytosis. The results conclude the specificity of SV119 conjugated dendrimers to cancer cells.

Task 2: To construct a therapeutic plasmid (p53) containing the heparanase promoter for breast cancer specific gene expression

The DH5 α bacterial cells containing GFP-p53 plasmid were obtained from Addgene (Plasmid 12091, Figure 5). The cells were grown in AccuGENE® LB broth (Lonza, Belgium) and plasmids were isolated using Qiagen plasmid Mini and Maxi kit (Qiagen Sciences, Maryland, USA). Plasmid concentration and purity, $A_{260}/A_{280} > 1.9$, was assessed using Nanodrop ND-1000 Spectrophotometer (Wilmington, DE). Plasmid integrity was confirmed by 1% agarose gel electrophoresis and stored at -20 °C until further use (Figure 6). Isolated DNA was digested using *Bam*HI and *Pst*II and p53 fragment was confirmed by electrophoresis (Figure 7).

DNA condensation ability of SV119-conjugated dendriplexes was evaluated by preparing dendriplexes at different charge ratios (N/P: 0.1 - 20) by mixing (pipetting and vortexing) dendrimer solution and the circular (non-linear) plasmid DNA in HEPES buffer (25 mM, 10 mM MgCl₂, pH 7.4) at room temperature for 30 min. Each sample was analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide (0.5 μ g/mL) at 75 V for one hour (Figure 8). To determine transfection efficiency, dendriplexes of SV119-conjugated PAMAMG4 dendrimers were prepared at charge ratios (N/P) 10, 20 and 40 by adding 1 μ g of GFP-p53 plasmid while vortexing in PBS for 30 min. MCF-7 and NCI/ADR-RES cells were plated at a density of 7×10^4 cells/well in a 12 well plate, grown for 24 hr at 37 °C in an atmosphere of 5% CO₂ and 95% relative humidity using RPMI 1640 medium (pH 7.4) having 10% fetal bovine serum. Dendriplexes at various N/P from 10-40 were added to the cells and incubated for 7 hr at 37 °C. The medium was removed, washed with PBS pH 7.4 and the cells were incubated with growth medium for another 48 hr. Levels of GFP in the transfected cells were detected and corrected for background fluorescence of the control cells using fluorescence-activated cell sorting (FACS) machine (BD FACSVantage™ Cell Sorter system, argon ion laser 488 nm). The transfection efficiency of pDNA-PAMAM-SV119 dendriplexes gradually increased with increase in charge ratio from N/P 10

(MCF-7: $13.65 \pm 1.25\%$, NCI/ADR-RES: 19.5 ± 2.3) to N/P 40 (MCF-7: $39.98 \pm 1.5\%$, NCI/ADR-RES: 39.74 ± 2.22) (Figure 9).

Task 3: To determine the therapeutic efficiency of dual targeting approach in various breast cancer cell lines

1. Preparation and characterization of the sigma ligand(s)-conjugated dendrimer-(GFP-p53) plasmid complexes
2. Gene transfection and apoptosis analysis will be performed by DAPI staining and western blotting in various breast cancer cell lines.

Transfection efficiency of the dendriplexes depend on their charge, stability and size. Profound zeta potential (+ve or -ve) values predict the stability and cell membrane interaction ability of the dendriplexes. Dendriplexes were prepared at different N/P ratios (1 - 10) between the SV119-conjugated PAMAMG4 dendrimers and the GFP-p53 plasmid. Briefly, GFP-p53 plasmid was mixed to PAMAMG4 dendrimer solution by pipetting, followed by incubation for 30 min with occasional vortexing. Zeta potential and size of the dendriplexes were measured by using ZetaPALS (Brookhaven Instruments Corporation, Holtsville, NY). All measurements were carried out on the dendriplexes with 5 $\mu\text{g/mL}$ plasmid DNA in HEPES buffer at pH 7.4. The Zeta potential increased while size of the dendriplexes decreased gradually with increase in N/P ratio in both PAMAMG4 and SV119-conjugated PAMAMG4 dendriplexes. At N/P ratio 10, size and zeta potential of the dendriplexes with PAMAMG4 and SV119-conjugated PAMAMG4 dendrimers was 112 ± 25 nm; 25.1 ± 5.2 mV and 128 ± 14 nm; 22.4 ± 3.8 mV, respectively.

Task 4: To determine the effect of targeted proapoptotic gene therapy on breast cancer drug resistance reversal

1. Induce doxorubicin drug resistance in various breast cancer (MCF-7) cells
2. Determine the efficacy of proapoptotic gene therapy followed by doxorubicin therapy on cell viability will be tested by MTT assay.

a) Determination of doxorubicin IC_{50} in MCF-7 and NCI/ADR-RES cells:

Method: Cytotoxicity of doxorubicin in MCF-7 and NCI/ADR-RES cells was evaluated by MTT assay. Briefly, MCF-7 and NCI/ADR-RES cells were plated at a density of 10×10^3 cells/well in a 96 well plate, grown for 24 hr at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity using RPMI 1640 Medium (pH 7.4) with 10% FBS. Doxorubicin at various concentrations (MCF-7: 10 nM – 80 nM and NCI/ADR-RES: 0.5 μg – 40 μg), were added to the cells and incubated for 72 hr at 37 °C. After 72 hr incubation, the medium was removed, washed with PBS pH 7.4 and 50 μL of MTT (0.5 mg/mL) was added and the cells were incubated approximately for 10 hr. The growth medium was removed and 150 μL of dimethyl sulfoxide (DMSO) was added to dissolve the MTT crystals and the optical density was read using a VMax Kinetic microplate reader (Molecular devices, Sunnyvale, CA) with 595 nm as excitation wavelength and 650 nm as the background. Viability of cells exposed to dendriplexes was expressed as a percentage of the viability of cells grown in the absence of dendriplexes. IC_{50} of doxorubicin was determined by GraphPad Prism version 5.03 software (GraphPad Software, Inc., La Jolla, CA).

Results: IC_{50} value of doxorubicin in MCF-7 was found to be 29.66 nM. In NCI/ADR-RES cells, IC_{50} value of doxorubicin could not be estimated in the concentration range used in the experiment. However, % viability of NCI/ADR-RES cells decreased from $80.01\% \pm 1.6$ (1 μM doxorubicin) to $67.09\% \pm 1.12$ (40 μM doxorubicin) (Figure 10).

b) Effect of dendriplex single treatment followed with doxorubicin:

Method: MCF-7 and NCI/ADR-RES cells were plated at a density of 10×10^3 cells/well in a 96 well plate, grown for 24 hr at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity using RPMI 1640 Medium (pH 7.4) with 10% FBS. Dendriplexes were prepared by complexing 0.5 μg of either GFP-p53 or GFP plasmid with the SV119-conjugated PAMAMG4 dendrimers at N/P 5 and 10, and were added to the cells followed by incubation at 37 °C for 7 hr. The medium was removed, washed with PBS pH 7.4 and the cells were incubated with growth medium containing doxorubicin at various concentrations (MCF-7: 0, 10 and 25 nM and NCI/ADR-RES: 0, 0.5 and 5 μM) for another 65 hr. The medium was removed, washed with PBS pH 7.4 and cell viability was assessed by MTT assay as described above. GFP plasmid served as plasmid control.

Results: IC₅₀ value of doxorubicin in MCF-7 decreased from 29.66 nM to 22.7 nM and 10.94 nM when used with the single transfection with GFP-p53 dendriplexes at N/P 5 and 10, respectively. No significant difference in % cell viability of NCI/ADR-RES cells was observed when they were transfected with GFP-p53 dendriplexes at all concentrations of doxorubicin used when compared to control plasmid (GFP plasmid) at N/P 5. However, significant difference in % cell viability was observed when cells were transfected at N/P 10 with GFP-p53 dendriplexes, where % cell viability decreased to 85.37±0.54, 72.83±1.69 and 63.49±1.69% as compared to control dendriplexes (GFP dendriplexes at N/P 10) showing 92.59±1.73, 82.32±1.72 and 70.68±0.54% with 0, 0.5 and 5 µM doxorubicin, respectively. Non-transfected cells showed % cell viability of 100±1.78, 80.76±0.42 and 72.83±2.83% with 0, 0.5 and 5 µM doxorubicin, respectively (Figure 11).

c) Effect of dendriplex multiple treatment followed with doxorubicin:

Method: MCF-7 and NCI/ADR-RES cells were plated at a density of 10x10³ cells/well in a 96 well plate, grown for 24 hr at 37 °C in an atmosphere of 5% CO₂ and 95% relative humidity using RPMI 1640 Medium (pH 7.4) with 10% FBS. 24 hr later (Day 1) GFP-p53 or GFP plasmid containing dendriplexes N/P 5 and 10 were added to the cells and incubated for 7 hr at 37 °C. The medium was removed, washed with PBS pH 7.4 and the cells were incubated with growth medium containing doxorubicin at various concentrations (MCF-7: 0, 10 and 25 nM and NCI/ADR-RES: 0, 0.5 and 5 µM) for another 17 hr. The procedure was repeated for two more days (Day 2 and Day 3). Finally, after 17 hrs incubation (3rd Day), the medium was removed, washed with PBS pH 7.4 and cell viability was assessed by MTT assay as described above.

Results: IC₅₀ value of doxorubicin in MCF-7 decreased from 29.03 nM to 22.73 nM and 12.55 nM when used with the multiple transfection with GFP-p53 dendriplexes at N/P 5 and 10 respectively. No significant difference in % cell viability of NCI/ADR-RES cells was observed when they were transfected with GFP-p53 dendriplexes at all concentrations of doxorubicin used when compared to control plasmid (GFP plasmid) at N/P 5. However, significant difference in % cell viability was observed when cells were transfected at N/P 10 with GFP-p53 dendriplexes, where % cell viability decreased to 79.15±1.34, 66.60±4.24 and 56.30±0.64% as compared to control dendriplexes (GFP dendriplexes at N/P 10) showing 92.28±6.43, 85.12±3.46 and 69.44±1.34% with 0, 0.5 and 5 µM doxorubicin, respectively. Non-transfected cells showed % cell viability of 100±4.46, 80.77±1.06 and 72.82±7.075 with 0, 0.5 and 5 µM doxorubicin, respectively (Figure 12).

d) Analysis of p53 expression by PCR:

Aim: p53 expression levels were determined in the single and multiple transfection studies using GFP-p53 dendriplexes (N/P 5 and 10), followed by treatment with doxorubicin at a concentration of 10 nM (MCF-7 cells) and 0.5 µM (RES/ADR-RES).

Method: GFP-p53 dendriplexes were prepared as described above. MCF-7 and NCI/ADR-RES cells were plated at a density of 5x10⁵ cells/25 cm² flask, grown for 24 hr at 37 °C in an atmosphere of 5% CO₂ and 95% relative humidity using RPMI 1640 Medium (pH 7.4) with 10% FBS. 24 hr later, both cell lines were transfected with GFP-p53 dendriplexes at N/P 5 and 10 for 7 hr followed by incubation with 10 nM (MCF-7 cells) and 0.5 µM (RES/ADR-RES) doxorubicin for 65 hr, separately. Multiple treatments were done as follows, for day 1: 7 hr transfection, washing the cells with PBS followed by incubation with doxorubicin for 17 hr, repeated for Day 2 and 3. Total RNA from the samples were isolated with RNeasy[®] mini kit (Qiagen sciences, MD, USA). The concentration of RNA was quantified using Nanodrop 1000 UV-Vis spectrophotometer (Nanodrop technologies, Inc, Wilmington, DE, USA). The first strand of cDNA was synthesized using 3 µg of RNA using RevertAid[®] premium first strand cDNA synthesis kit (Fermentas Inc., Glen burnie, MD, USA) as per manufacturer's protocol. Purity of the total RNA for DNA contamination was assessed by performing no reverse transcriptase control experiments with PCR using GAPDH primers provided in RevertAid[®] premium first strand cDNA synthesis kit (Fermentas Inc., Glen burnie, MD, USA). The cDNA thus obtained was diluted at 1:100 ratio with deionized water for PCR for p53 expression. The sequences for forward and reverse human p53 (Gene bank accession # AB082923) primers were 5'- TTG GTC GGT GGG TTG GTA GTT TCT-3' and 5'- ACC AAG AGG TTG TCA GAC AGG GTT - 3' respectively. Primers were purchased from Integrated DNA technologies, Iowa, USA. PCR was performed using 50 µL of reaction volume containing 2 µL of 1:100 diluted cDNA, 0.2 mM MgCl₂, 0.5 µM primers, and 2.5 units of Taq DNA polymerase (Fermentas Inc., Glen burnie, MD, USA). The reaction was run for 35 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72 °C for 1 min. The PCR products were electrophoresed through 1% agarose gel containing ethidium bromide. The image capturing and volume analysis of bands was done using Quantity one[®] software (Bio Rad, Hercules, CA, USA).

Results: As shown in Figure 13, p53 expression levels in the cells with multiple transfections were significantly higher than the single transfection, with no significant difference between MCF-7 and doxorubicin resistant breast cancer cell line. Moreover, the doxorubicin resistant cells appear to be sensitized to doxorubicin therapy following p53 transfection. Though a synergistic effect was observed between p53 and doxorubicin treatments, the results are not as high as expected. The observed results may be accounted for i) the lack of sustenance in p53 expression, as non-viral vector is used for transfection, ii) possible mutation of the transduced p53 over a period of time.

Key research accomplishments: For the first time novel sigma ligand conjugated PAMAM dendrimers have been synthesized and their specificity towards breast cancer cells was demonstrated.

Reportable outcomes: Proapoptotic gene therapy sensitizes the resistant breast cancer cells chemotherapy.

Conclusion: Sigma receptor ligand-conjugated PAMAM dendrimers have demonstrated specificity for various cancer cell lines including breast cancer. The therapeutic plasmid, GFP-p53, was efficiently condensed with PAMAM dendrimers at charge ratio (N/P)>1 and the complexes were stable in serum. No significant difference in transfection efficiency of the SV119-conjugated dendrimers was seen between MCF-7 and NCI/ADR-Res cell lines. IC₅₀ of doxorubicin significantly decreased with prior transfection with p53 in both MCF-7 and NCI/ADR-Res cell lines. A synergistic effect was seen between p53 transfection and chemotherapy in both the MCF-7 and doxorubicin-resistant cell lines. The present study validates our hypothesis that proapoptotic gene (p53) therapy sensitizes doxorubicin-resistant cells to doxorubicin therapy. Further investigation may be warranted to determine the therapeutic efficacy of a combination gene therapy in sensitizing the drug resistant breast cancer cells.

References:

1. Stoff-Khalili MA, Dall P, Curiel DT. Gene therapy for carcinoma of the breast. *Cancer Gene Ther.*, 2006, 13(7), 633-647.
2. Zeng C, Vangveravong S, Xu J, Chang KC, Hotchkiss RS, Wheeler KT, Shen D, Zhuang ZP, Kung HF, Mach RH. Subcellular localization of sigma-2 receptors in breast cancer cells using two-photon and confocal microscopy. *Cancer Res.* 2007, 67(14):6708-16.
3. Tu Z, Dence CS, Ponde DE, Jones L, Wheeler KT, Welch MJ, Mach RH. Carbon-11 labeled sigma2 receptor ligands for imaging breast cancer. *Nucl Med Biol.* 2005, 32(5):423-30.
4. Breidenbach M, Rein AT, Schondorf T, Khan KN, Herrmann I, Schmidt T, Reynolds PN, Vlodavsky I, Havib YS, Curiel DT. A new targeting approach for breast cancer gene therapy using Heparanase promoter. *Cancer Lett.*, 2006, 240, 114-122.
5. Mukherjee A, Prasad TK, Rao NM, Banerjee R. Haloperidol associated stealth liposomes. A potent carrier for delivering genes to human breast cancer cells. *J. Biol. Chem.*, 2005, 280(16):15619-15627.
6. Ndoye A, Merlin JL, Leroux A, Dolivet G, Erbacher P, Behr JP, Berg K, Guillemin F. Enhanced gene transfer and cell death following p53 gene transfer using photochemical internalisation of glucosylated PEI-DNA complexes. *J Gene Med.* 2004;6(8):884-94.
7. Vinyals A, Peinado MA, Gonzalez-Garrigues M, Monzó M, Bonfil RD, Fabra A. Failure of wild-type p53 gene therapy in human cancer cells expressing a mutant p53 protein. *Gene Ther.* 1999;6(1):22-33.
8. Bassett EA, Wang W, Rastinejad F, El-Deiry WS. Structural and functional basis for therapeutic modulation of p53 signaling. *Clin Cancer Res.* 2008;14(20):6376-86.

Appendix I: Figures 1-13

Appendix I:

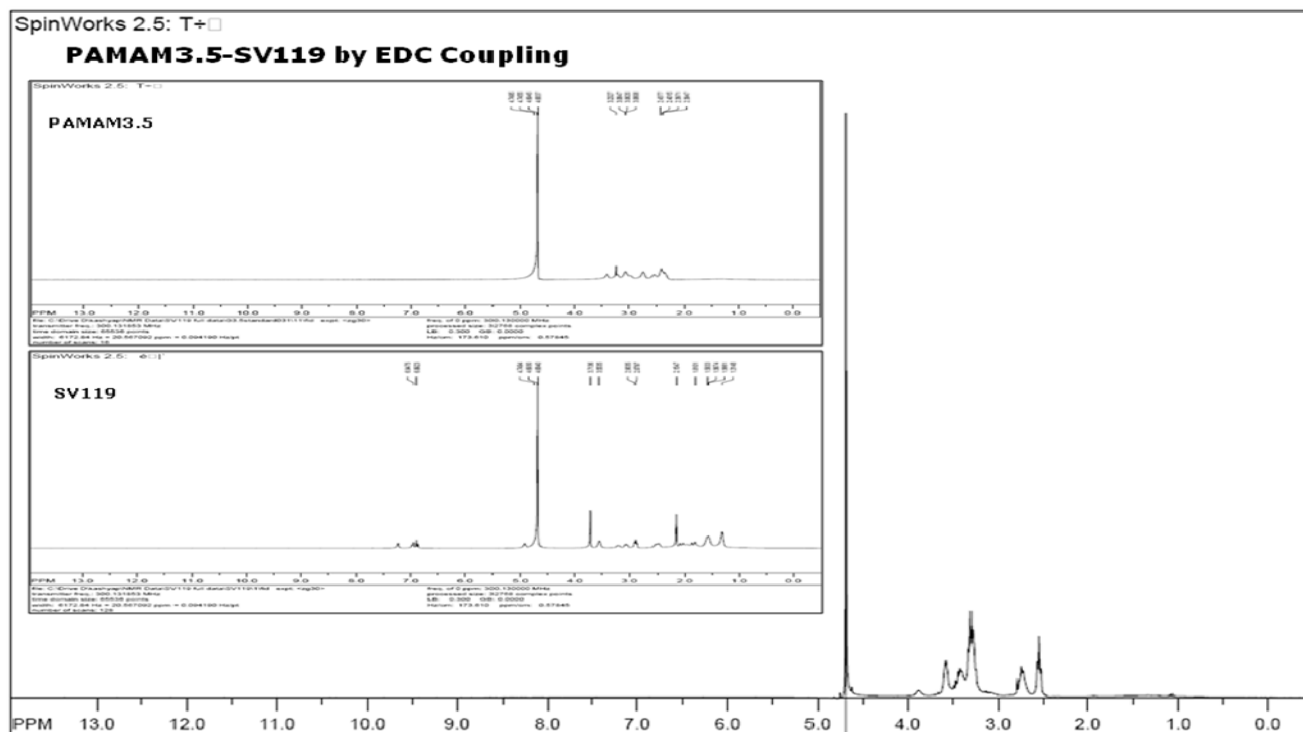


Figure 1: ^1H NMR data of SV119 conjugated PAMAM dendrimers 3.5 by EDC coupling.

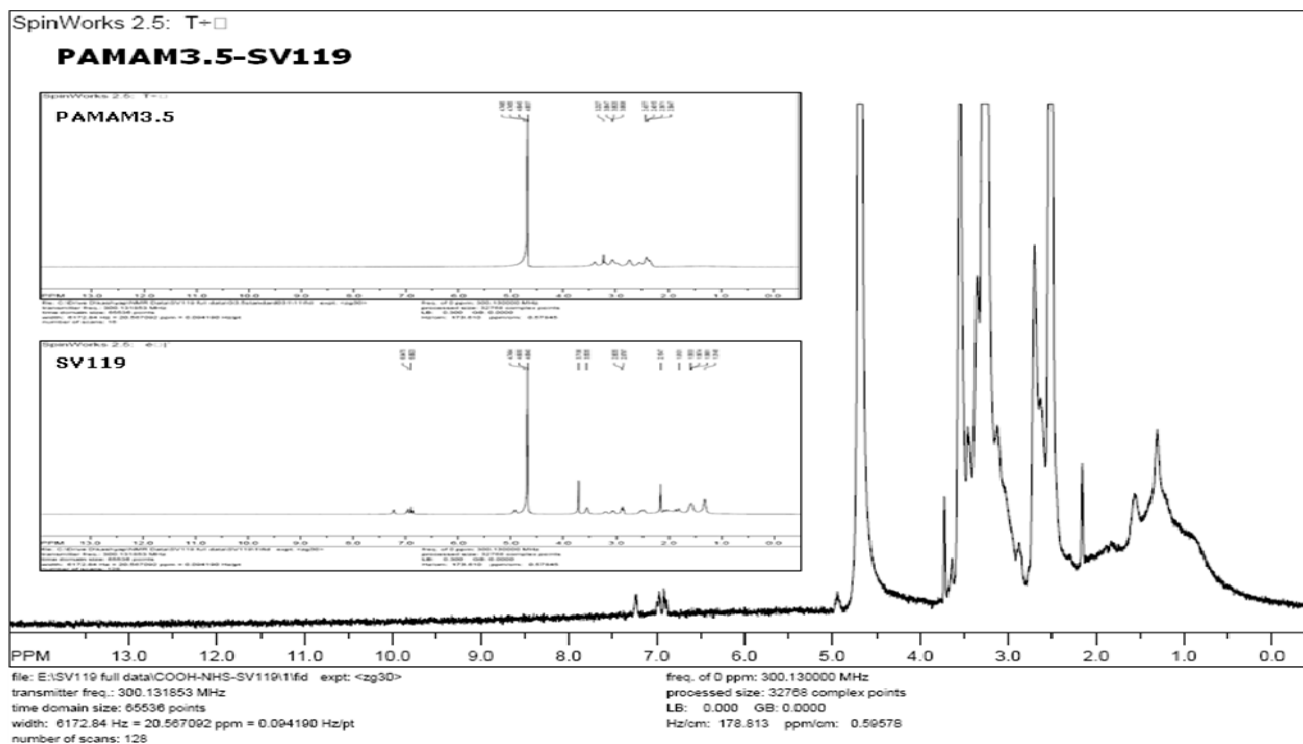


Figure 2: ^1H NMR data of SV119 conjugated PAMAM dendrimers 3.5 by NHS ester formation.

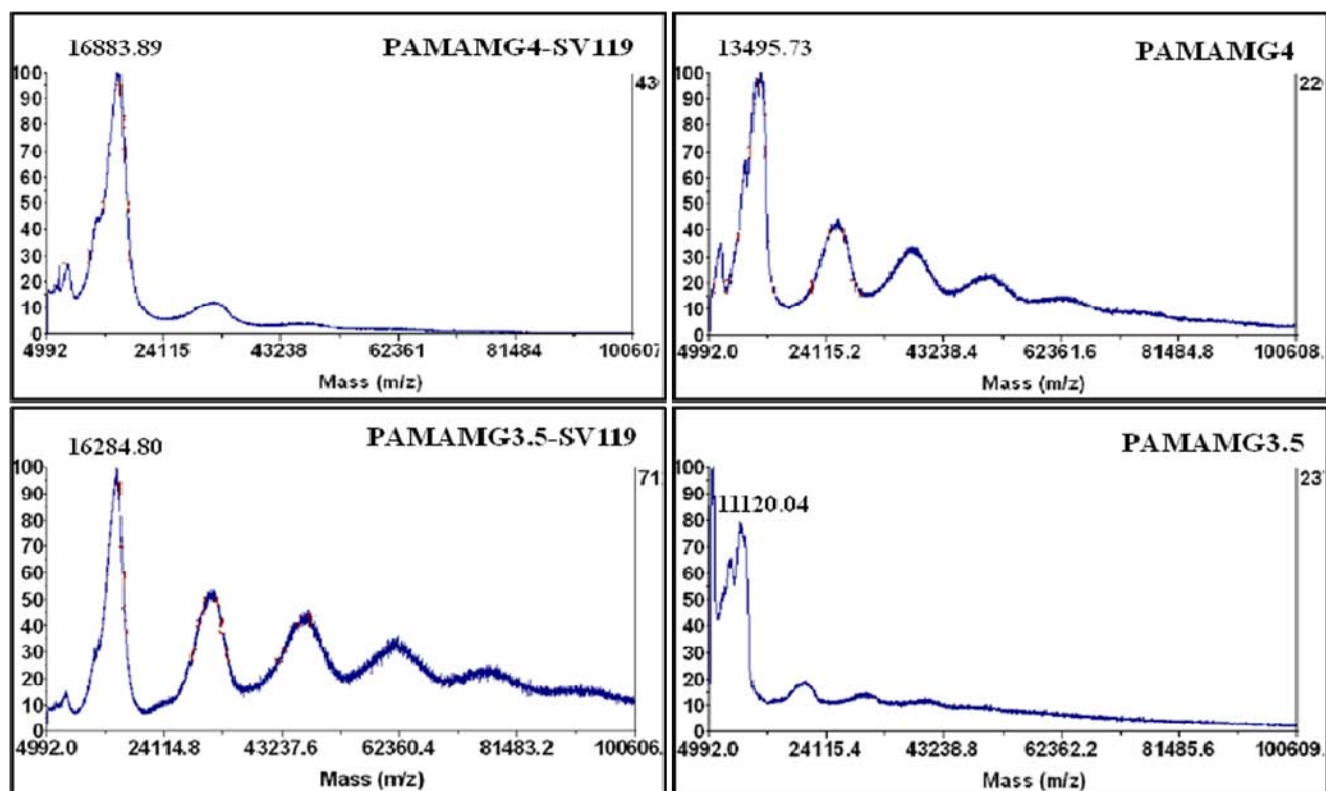


Figure 3: MALDI-TOF spectra of SV119 conjugated PAMAMG3.5 and PAMAMG4 dendrimers prepared by NHS ester formation.

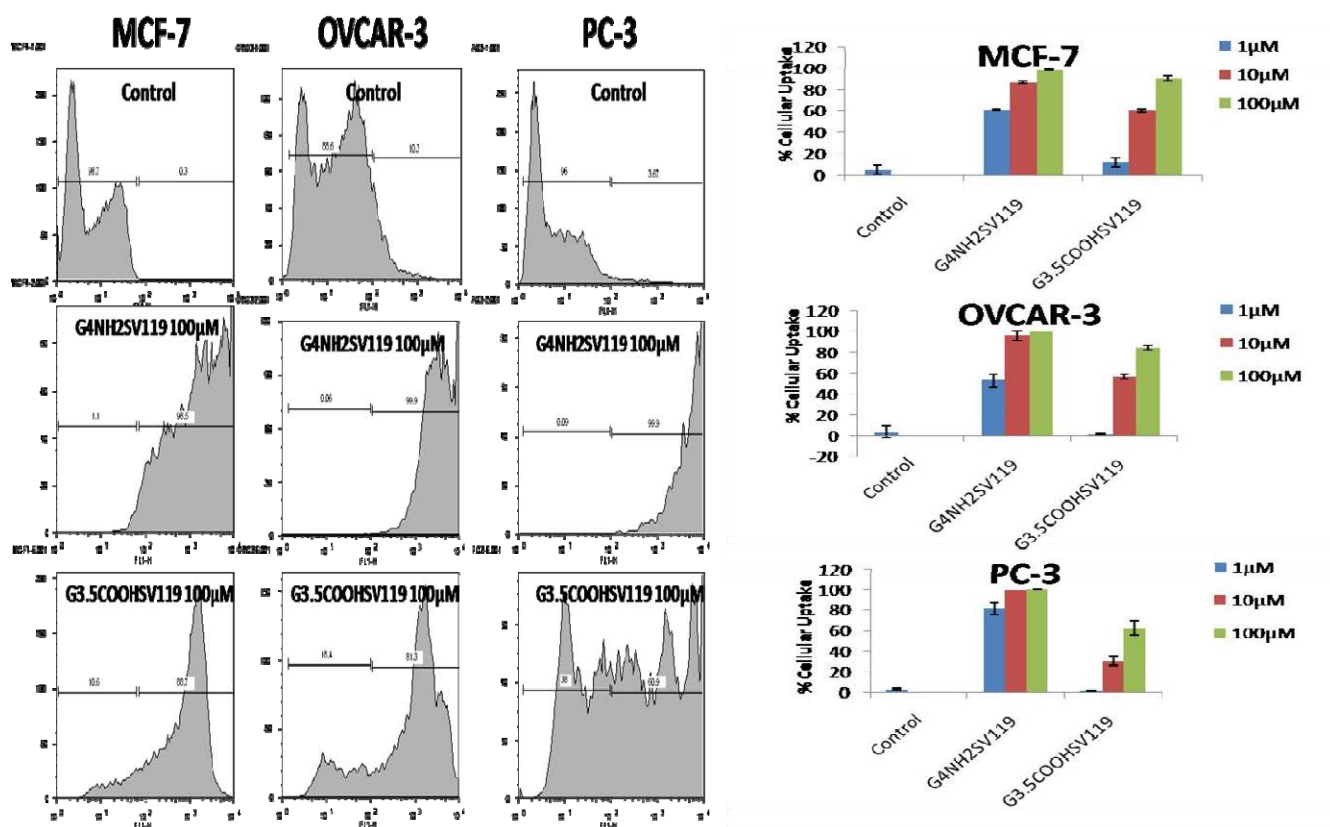


Figure 4: Cellular uptake and cancer cell-specificity of SV119 conjugated dendrimers.

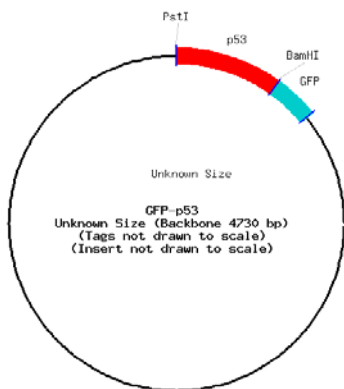


Figure 5: Addgene, Plasmid# 12091.

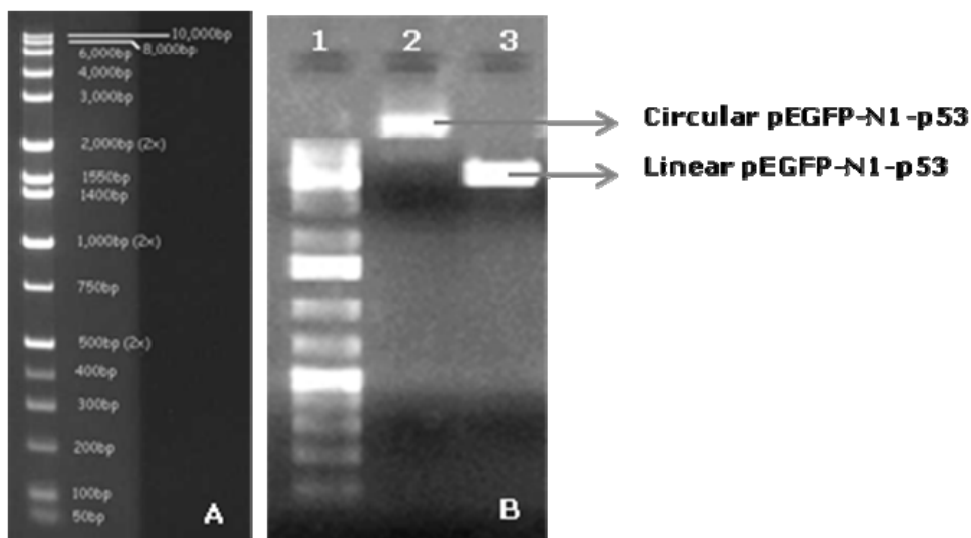


Figure 6: Closed circular and linear pEGFP-N1-p53. **[A]** DNA Ladder (Minnesota Molecular, Inc., Minneapolis, MN). **[B] Lane 1:** DNA Ladder, **Lane 2:** Purified circular plasmid (Addgene# 12091), **Lane 3:** Linear plasmid DNA obtained after digestion with *BamHI* (Fermentas Inc., Glen Burnie, MD).

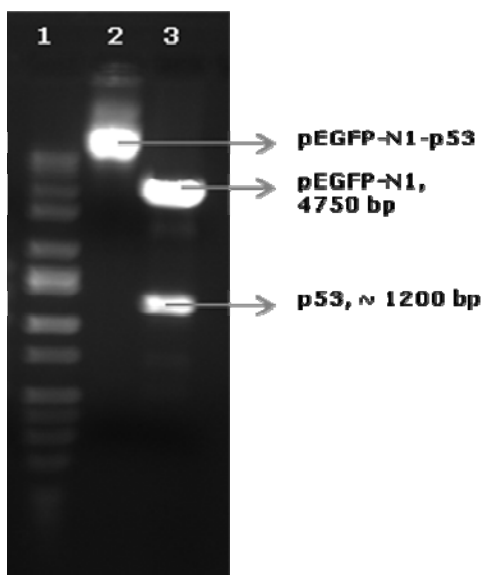


Figure 7: Restriction digestion of pEGFP-N1-p53 Lane 1: DNA Ladder, **Lane 2:** Purified plasmid, **Lane 3:** Plasmid digestion using *BamHI* and *PstI* (Fermentas Inc., Glen Burnie, MD)

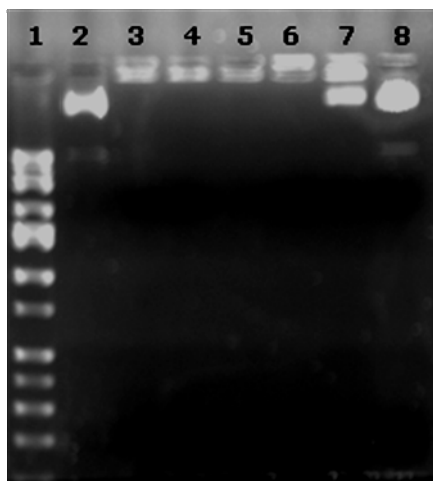


Figure 8: Agarose gel electrophoresis of dendriplexes

Lane 1: DNA Ladder, Lane 2: Purified plasmid, Lane 3-8: Dendriplexes at N/P 20, 10, 5, 1, 0.5, 0.1.

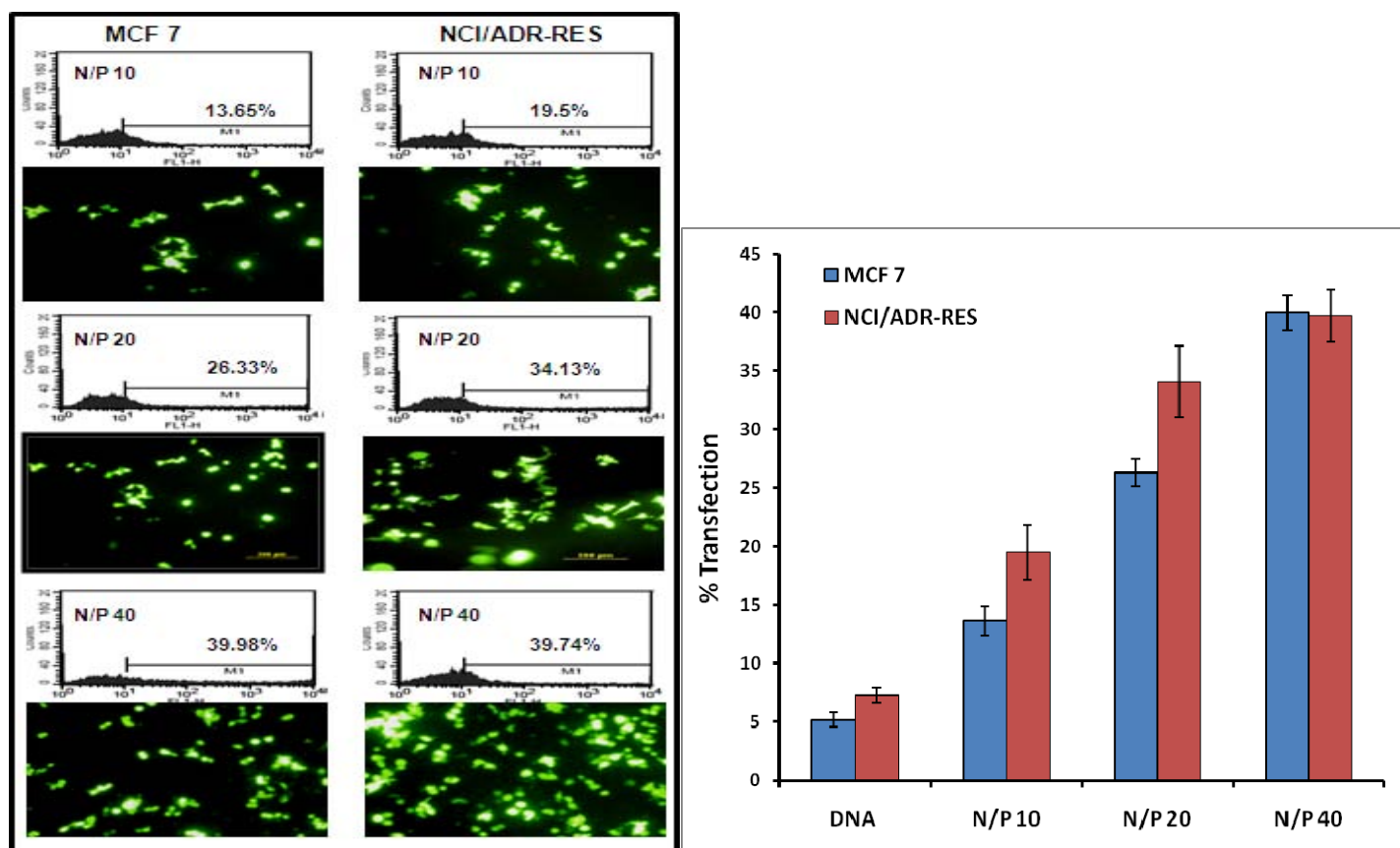


Figure 9: Gene transfection by SV119-conjugated PAMAMG4 dendriplexes in MCF-7 and NCI/ADR-RES cells. Cells were transfected with GFP-p53 plasmid condensed with SV119-conjugated PAMAMG4 dendrimers at N/P 10, 20 and 40. **Left Panel:** Histograms were generated using CellQuestPro software and corresponding fluorescent micrographs obtained by fluorescent microscopy of expressed GFP in the above mentioned cell lines. **Right Panel:** Comparative flow cytometry analysis of SV119-conjugated PAMAMG4 dendriplexes in the above said cell lines. Transfection efficiency was determined using CellQuestPro software. Bars represent number of GFP positive cells (%). Each data point in line represents the mean \pm standard deviation ($n = 3$). Percent transfection increased with increased N/P in both cell lines tested ($n = 3$, $p < 0.05$).

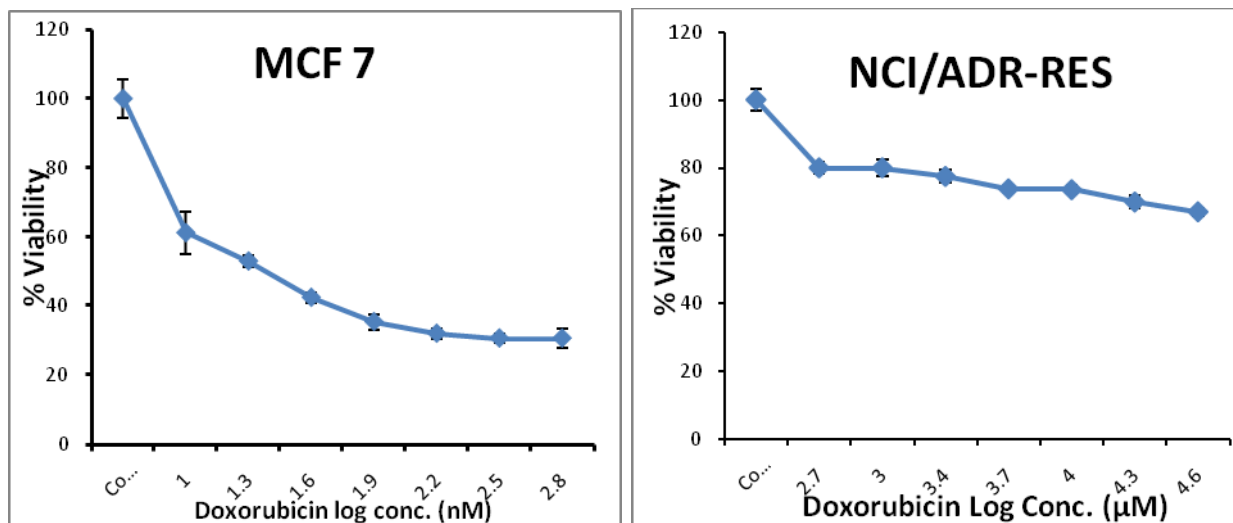


Figure 10: Determination of doxorubicin IC₅₀ in MCF-7 and NCI/ADR-RES cells. 10x10³ cells/well were plated in a 96 well plate in growth medium. 24 hr later, cells were washed and indicated amount of doxorubicin was added to the cells in growth medium and cells were incubated for another 72 hr. Following 72 hr incubation, cells were washed with PBS and % Viability was analysed using MTT assay. Each data point represents the mean ± standard deviation (n = 7).

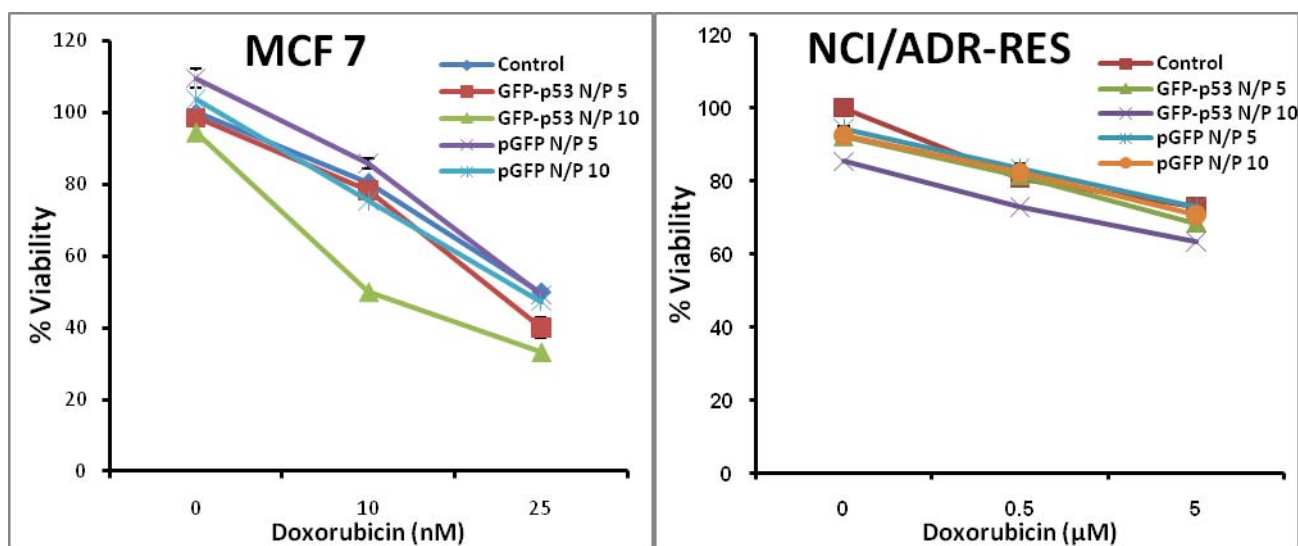


Figure 11: Synergistic effect determination of dendriplex single treatment followed with doxorubicin. 10x10³ cells/well were plated in a 96 well plate in growth medium. 24 hr later, cells were washed with PBS and were transfected with dendriplexes for 7 hr. Following transfection, cells were washed with PBS and were grown for another 65 hr in media containing doxorubicin. Following 65 hr incubation, cells were washed with PBS and % Viability was analyzed using MTT assay. Each data point in line represents the mean ± standard deviation (n = 3).

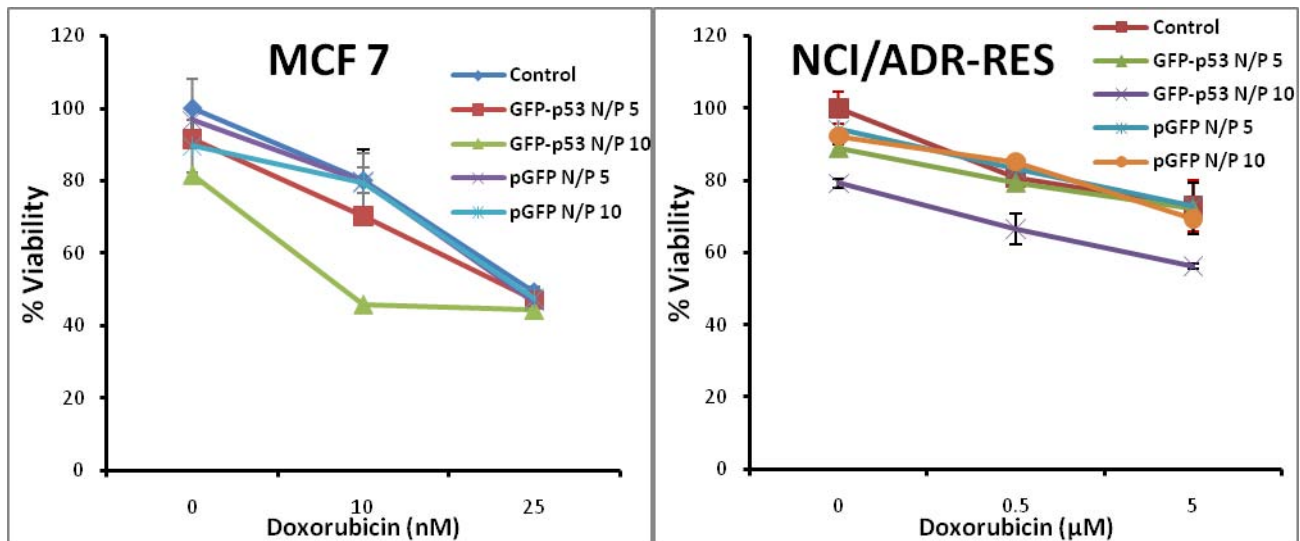


Figure 12: Synergistic effect determination of dendriplex multiple treatments followed with doxorubicin. 10×10^3 cells/well were plated in a 96 well plate in growth medium. 24 hr later, cells were washed with PBS and were transfected with dendriplexes for 7 hr (Day 1). Following transfection cells were washed with PBS and were let to grow for another 17 hr in media containing doxorubicin (Day 1). The method was repeated on Day 2 and 3. Following 3rd Day, cells were washed with PBS and % Viability was analyzed using MTT assay. Each data point in line represents the mean \pm standard deviation (n = 3).

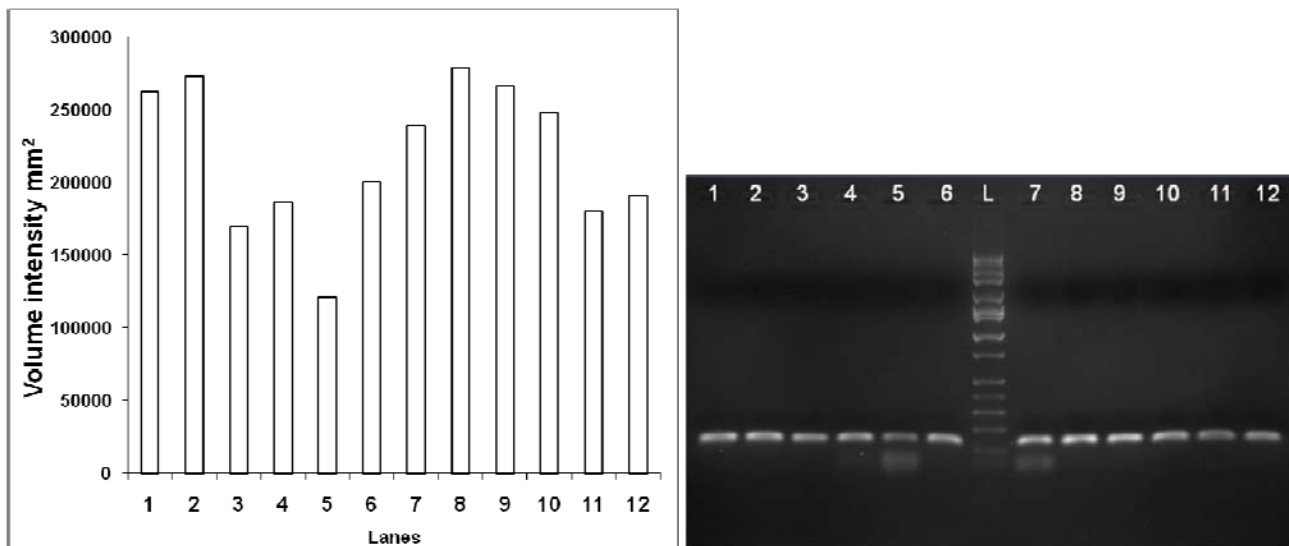


Figure 13: Analysis of treated samples for p53 expression by PCR. MCF-7 and NCI/ADR-RES cells were plated at a density of 5×10^5 cells/25 cm² flask, grown for 24 hr. 24 hr later, both cell lines were transfected with dendriplexes for 7 hr followed by incubation with 10 nM (MCF-7 cells) and 0.5 μM (RES/ADR-RES) doxorubicin for 65 hr. Multiple treatments were done for Day 1: 7 hr transfection, followed by incubation with doxorubicin for 17 hr, repeated for Day 2 and 3. After completion of treatments, total RNA from the samples were isolated, quantified, cDNA was synthesized and p53 gene was amplified by PCR. The PCR products were subjected to 1% agarose gel electrophoresis. The image capturing and volume analysis of bands was done using Quantity one[®] software (Bio Rad, Hercules, CA, USA). Left to right: **Lane 1-6** represents samples of MCF-7, while **Lane 7-12** represents samples of NCI/ADR-RES, **1 and 7**: multiple dendriplex (N/P 10) treatment with Adriamycin; **2 and 8**: multiple dendriplex (N/P 5) treatment with Adriamycin; **3 and 9**: single dendriplex (N/P 10) treatment with Adriamycin; **4 and 10**: single dendriplex (N/P 5) treatment with Adriamycin; **5 and 11**: Adriamycin treatment; **6 and 12**: controls; L: DNA Ladder.